

Posters

Muscle Regulation I

1157-Pos Board B1

Purification and Proteomic Analysis of Human Membrane Lipid Rafts and Their Importance in Ca^{2+} -sensitization of Vascular Smooth Muscle (VSM) Contraction

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Hypercholesterolemia is a major risk factor for cardiovascular events. Among them, abnormal VSM contractions such as vasospasm are caused by Ca^{2+} -sensitization of VSM contraction. However, the relationship between cholesterol (CHOL) and the VSM Ca^{2+} -sensitization has not been clarified yet. Our recent studies showed that a sphingosylphosphorylcholine (SPC)/Src family tyrosine kinase (Src-TK) / Rho-kinase (ROK) pathway mediates the Ca^{2+} -sensitization, and SPC indeed induces severe vasospasm in vivo. We found that serum CHOL potentiates the SPC/Src-TK/ROK pathway leading to Ca^{2+} -sensitization in both human and rabbit: the extent of SPC-induced Ca^{2+} -sensitization correlated well with total CHOL and LDL-CHOL, but inversely correlated with HDL-CHOL. Moreover, the depletion of CHOL by β -cyclodextrin destroyed CHOL-enriched membrane lipid rafts and abolished the SPC-induced ROK translocation and Ca^{2+} -sensitization, suggesting the SPC-induced translocation of ROK to lipid rafts. Taken together, we suggested that not only CHOL, but also lipid rafts mediates the SPC-induced Ca^{2+} -sensitization. Understanding the mechanism(s) by which lipid rafts promotes Ca^{2+} -sensitization in human VSM requires the elucidation of lipid raft protein composition. As a first step, we succeeded for the first time in purifying lipid rafts from human VSM by sucrose density gradient ultracentrifugation, which were confirmed by western blot of raft marker proteins such as caveolin-1 and flotillin-1. Subsequently, using mass spectrometry (MALDI TOF-MS), proteomic analysis was performed to compare the protein compositions between lipid and non-lipid raft membrane fractions. The ongoing studies have identified so far previously unreported novel raft-localized proteins, in addition to the known proteins, including lipid- or GPI-anchored proteins and membrane proteins. We are attempting to accumulate functional data to suggest that some novel signaling molecules contribute to an SPC/Src-TK/ROK pathway leading to the VSM Ca^{2+} -sensitization.

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Phosphorylation-Induced Structural Changes in Smooth Muscle Regulatory Light Chain

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We are using site-directed spectroscopic labeling, fluorescence, and molecular dynamics simulations to define the phosphorylation-induced structural transition in smooth muscle myosin regulatory light chain (RLC). Smooth muscle is activated through phosphorylation of Ser 19 on RLC, but the N-terminal 24 amino acids of RLC do not appear in any crystal structure. EPR experiments (Nelson et al., 2005) have shown that phosphorylation induces a disorder-to-order transition within the N-terminal phosphorylation domain of the RLC, in which increased helical ordering relieves inhibitory head-head interactions. To define this structural change in atomic detail, we are combining molecular dynamics simulations with spectroscopic distance constraints. Simulations on the unphosphorylated 25-residue N-terminal fragment of the RLC reveal a disordered region in T9-K12, while the phosphorylated N-terminal domain maintains strong α -helicity over the same residues. This disorder-to-order transition is regulated by delicate balance between enthalpy and entropy involving R16. The same disorder-to-order transition has been observed by both simulations extended to include the entire RLC in complex with a portion of the myosin heavy chain. Furthermore, we have employed FRET distance measurements on di-Cys mutant RLC exchanged onto S1 and HMM, to provide geometric constraints for the simulations. This allows determination of both structure and dynamics of the regulatory domain in the absence and presence of phosphorylation. This work was supported by grants from NIH (AR32961, AR07612) and the Minnesota Supercomputing Institute. We thank Igor Negrashov for excellent technical assistance.

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The Involvement of Fyn Tyrosine Kinase in the Signal Transduction of Ca^{2+} sensitization of Vascular Smooth Muscle Contraction Mediated by Rho-kinase

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Rho-kinase (ROK)-mediated Ca^{2+} sensitization of vascular smooth muscle (VSM) contraction plays a critical role in abnormal VSM contraction such as vasospasm. Previously we found that sphingosylphosphorylcholine (SPC) induces the ROK-mediated Ca^{2+} sensitization through the activation of Src family tyrosine kinase (Src-TK) independently of a protein kinase C pathway. Since western blot analysis showed the presence of Fyn and c-Src among Src-TKs in VSM, we aimed to clarify which Src-TK is truly involved in the Ca^{2+} sensitization mediated by the SPC/ROK pathway. Immunofluorescent study showed that SPC induced the translocation of Fyn, but not c-Src, to plasma membrane in cultured VSM cells, which was blocked by eicosapentaenoic acid, a specific inhibitor of the SPC-induced Ca^{2+} sensitization. The siRNA-mediated knockdown of Fyn diminished the SPC-induced contraction remarkably in cultured VSM cells. In β -escin-permeabilized VSM strips, constitutively-active Fyn, which was produced by a baculovirus expression system, induced the Ca^{2+} sensitization, which was blocked by Y27632 (a ROK inhibitor). Dominant-negative Fyn blocked the Ca^{2+} sensitization induced by SPC, GTP γ S (a direct activator of G-protein), and a GPCR agonist + GTP. Overexpression of constitutively-active and dominant-negative Fyn induced contraction and relaxation of cultured VSM cells, respectively. Furthermore, in order to identify the target(s) of Fyn, tyrosine-phosphorylated proteins were immunoprecipitated from the VSM cells which were treated with or without SPC. Subsequently, nanoflow liquid chromatography-tandem mass spectrometry (nanoLC-MS/MS) was used to identify the SPC-induced tyrosine-phosphorylated and tyrosine-dephosphorylated proteins and their phosphorylation sites. These findings clearly indicate that Fyn tyrosine kinase plays an essential role in the ROK-mediated Ca^{2+} sensitization of VSM contraction. Functional proteomic approach has been useful to reveal possible targets of Fyn, and their functional roles are currently under investigation.

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Simultaneous Measurement Of Force And Fluorescence In Single Guinea-pig Cardiac Myofibrils

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Kinetic studies of force activation and relaxation in the single myofibril preparation have enhanced our understanding of mechanisms that underlie regulation of muscle force. Likewise, stop-flow and steady-state measurement using fluorescently labeled TnC upon Ca^{2+} activation in reconstituted protein systems have provided data regarding the kinetics of thin filament regulation. However, to develop a detailed understanding and fully characterize muscle regulation, an integration of mechanical, biochemical, and structural information is necessary. We present the development of a new apparatus which allows simultaneous measurement of fluorescent probe signals from labeled contractile proteins (such as TnC), and force kinetics under active isometric force development to allow direct correlation between these parameters in a single myofibril. Single guinea-pig cardiac myofibrils were prepared in relaxing solution (PCa8.0) by mechanical homogenization; endogenous TnC was exchanged for recombinant mutant labeled human TnC (2.5 mg/ml; overnight 4°C; Alexa-350 label conjugated to cys-84). Myofibrils were attached to glass micro-tools with one serving as a cantilever of known stiffness to assess force (painted black for improved contrast) and the other attached to a high speed motor. Myofibrils were activated with a Ca^{2+} pulse delivered via a double barreled perfusion pipette attached to a stepper motor (~5 ms). Excitation UV-light (270-350 nm), chopped at 52 kHz using a photo-elastic modulator, was projected (400nm DC mirror) onto the attached myofibril. Force probe deflection was measured in bright-field using red light (670nm) while fluorescence emission was measured (420nm LP filter) using a photomultiplier. Data was sampled by a high-speed (2MHz) A/D converter and demodulated using custom designed software. Preliminary results indicate that using this apparatus and techniques, ultra-low intensity TnC fluorescence signals can be obtained at high temporal resolution in parallel with bright-field optical measurement of force development.

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Activation and Relaxation Kinetics in Isolated Guinea-Pig Myofibrils: Impact of Sarcomere Length

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Myofilament length dependent activation is a universal property of striated muscle that is most pronounced in myocardium. The mechanisms that underlie length dependency are incompletely understood. Accordingly, here we tested the hypothesis that sarcomere length (SL) affects the rate of force activation and/or force relaxation in response to a saturating pulse of Ca^{2+} . Isolated skinned guinea-pig myofibrils were attached to glass micro-tools positioned on the stage of an inverted microscope (15°C); one probe functioned as a force